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A Role for MEK-Interacting Protein 1 in Hormone Responsiveness of ER Positive Breast Cancer Cells

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14. ABSTRACT

We have identified a novel role for the small scaffold protein MP1 in the survival of ER-positive breast cancer cell lines. Blocking MP1 expression using siRNA leads to apoptotic cell death in ER-positive MCF-7, LCC9 and T47D cells, but not in ER-negative MDA-MB-231, SKBr3 and BT-549 cell, or in non-tumorigenic 184B5 cells. Inhibition of MP1 expression in MCF-7 cells resulted in decreased ER expression and activity, and decreased AKT phosphorylation. We therefore hypothesize that a loss of pro-survival signaling from one or both of these molecules may be responsible for the cell death observed upon MP1 knockdown. In addition, we have obtained preliminary evidence suggesting that inhibiting MP1 expression in ER-positive cells leads to decreased expression of several integrins, which may also contribute to the cell detachment and/or death observed. The current targeted therapies for ER-positive tumors (antiestrogens and aromatase inhibitors) induce a cell cycle arrest, but not rapid cell death. Our results suggest that MP1or one of the pro-survival pathways that it regulates could provide novel targets for the treatment of ER-positive breast tumor. If such treatments result in cell death, they may provide advantages over current therapies such as decreased treatment time.

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Introduction

Our original proposal contained preliminary data suggesting that estrogen receptor alpha (ER) is present in a complex with the small scaffold protein Mek Partner-1 (MP1) in human breast cancer cell lines, and that overexpression of MP1 via transient transfection increases ER's transcriptional activity. MP1 is a widely expressed scaffold protein that interacts with several intracellular kinases that are known to impact ER function, including MEK, ERK and PAK1. MP1 was first identified as a protein that binds to ERK and MEK, and that potentiates MAPK signaling [1]. It also binds to active PAK1 at the plasma membrane, and integrates PAK1 and Rho signaling [2]. Knockdown of MP1 inhibits spreading of fibroblasts on fibronectin [2], and also results in decreased migration of human prostate cancer epithelial cells on fibronectin [3]. Surprisingly, neither ERK nor PAK1 phosphorylation was dramatically altered when MP1 expression was inhibited in prostate cancer epithelial cells. The decreased migration was, however, correlated with decreased paxillin expression and with changes in the number and turnover of focal adhesions at the migratory edge. Thus, one function of MP1 is related to cell attachment, spreading and migration.

Because of our preliminary findings suggesting an interaction between ER and MP1, we proposed that MP1 would play an important function in ER-positive breast cancer cells. The original aims of our proposal were **1: To test the hypothesis that MP1 is required for ER function and proliferation in human breast cancer cells, and 2: To characterize the subcellular localization and protein composition of ER/MP1 complexes.** In our previous annual reports, we described experiments using transfection of small interfering (si) RNAs to inhibit MP1 expression and examine the effects on ER activity and cell proliferation. Surprisingly, we found that rather than decreasing proliferation, inhibiting MP1 expression led to rapid cell detachment and death. Our conclusions from the first two years of the project can be briefly summarized as follows. 1) Inhibition of MP1 expression led to cell detachment and apoptosis of ER-positive, but not ER-negative, breast cancer cell lines. 2) The cell death observed in ER-positive cell lines was associated with an approximate 2-3 fold decrease in ER expression and transcriptional activity. Our previous reports also described the difficulties that we experienced expressing MP1 in stably transfected cell lines, and in carrying out proposed co-immune-precipitation experiments. Our progress in the third year of the project is reported below.

Body: Progress on Each Task in Approved Statement of Work.

Task 1: Test hypothesis that MP1 expression is required for ER's transcriptional activity and proliferation of human breast cancer cells.

<u>Is MP1 expression required in non-tumorigenic mammary epithelial cells?</u> Our previous experiments demonstrated that MP1 was required for cell survival of ER-positive, but not ER-negative breast cancer cell lines. To investigate its role in non-tumorigenic mammary epithelial cells, both control and MP1 siRNA was transfected into 184B5, an immortalized but non-tumorigenic human mammary epithelial cell line. As shown in Figure 1, there was no significant increase in cell detachment or cell death as a result of MP1 knockdown. Additional experiments were carried out with a second non-tumorigenic cell line, MCF10A, and similar results were

obtained (data not shown). Neither 184B5 nor MCF10A express high levels of ER, and we are unaware of any non-tumorigenic human mammary epithelial cell lines that do. We have therefore been unable to address the question of whether MP1 expression is required in non-transformed, ER-positive mammary epithelial cells.

The effects of MP1 knockdown on cell survival pathways in ER-positive breast cancer cells. We have initiated experiments to identify the pathways that are contributing to cell death when MP1 expression is inhibited in the ER-positive MCF-7 breast cancer cell line. The hypothesis of these experiments is that inhibiting MP1 expression results in decreased pro-survival signaling. As an initial test of this hypothesis, we measured the levels of both total and activated (phosphorylated) ERK, JNK, and AKT1 in cells transfected with control or MP1 siRNA. No differences in ERK or JNK phosphorylation were detected between control and MP1 siRNA transfected cells (data not shown). However, as shown in Figure 2A, there was an approximate two fold decrease in phospho-AKT1 upon MP1 knockdown. Although this effect seems modest given the dramatic cell death phenotype observed, we believe that it may be an underestimate. The cell extracts used in these experiments were prepared at 24 and 48 h after transfection, and by these time points a significant number of cells treated with MP1 siRNA had died and/or detached from the plates. Although we collected both attached and detached cells, we have found that the detached cells contain very little protein. The majority of the protein in these extracts may therefore come from the remaining attached cells, which are likely enriched for those with less efficient MP1 knockdown.

We have initiated experiments to investigate if the decrease in AKT phosphorylation/activity is responsible for the cell death phenotype observed. MCF-7 cells were transfected with a pBabe vector, or with pBabe encoding a constitutively active (myristilated) form of AKT1 that is also Flag-tagged (Myr-Flag-AKT). Stable transfectants were selected, and screened for expression of the Flag-tagged protein, as well as the phosphorylated (active) form of AKT. As shown in Figure 2B, we have identified several clones that are expressing the transfected gene. In addition to these clonal cell lines, we have also established several pools of transfected cells. We are currently testing whether these transfectants are resistant to cell death induced by MP1 knockdown.

We have also conducted preliminary experiments with PCR arrays to identify additional pathways that might be involved in the cell death response to MP1 knockdown. We are still in the process of carrying out these experiments and verifying the results using real time RT/PCR and western blotting. However, one particularly interesting preliminary finding is that there are 3-5 fold decreases in the expression of several integrins (including alpha2, alpha3, beta 1 and beta5) in MP1 siRNA treated cells compared to control siRNA treated cells. Since integrins are involved in both cell attachment and survival, decreases in their expression may contribute to the detachment and/or cell death observed.

Effects of MP1 on cell migration. In our previous progress report we described experiments indicating that overexpression of MP1 via transient transfection led to increased ER expression and activity. We have now carried out additional experiments to investigate the effect of MP1 overexpression on the phenotype of MCF-7 cells. As shown in Figures 3 and 4, transient overexpression of MP1 led to increased cell migration and invasion in Boyden chamber assays. Since overexpression of MP1 increased migration of the weakly migratory MCF-7 cells, we also investigated the effects of inhibiting its expression in the highly migratory, ER-negative MDA-

MB-231 breast cancer cell line. As shown in Fi gure 5, inhibiting MP1 express ion had no effect on m igration of MDA- MB-231 cells. Thus, as in the case of cell death, the effect of MP1 on migration m ay be specif ic to E R-positive cell lines. This possib ility is cur rently bein g investigated using additional cell lines.

Task 2: Determine the subcellular localization of ER/MP1 complexes.

In our original application, we proposed to identify the subcellular localization of ER/MP1 complexes by isolating various fractions (m embrane, cytosol, nucleus, etc.) and then analyzing these fractions for ER/MP1 com plexes by co-im munoprecipitation experiments. As described in our first previous reports, we ha ve been unable to reproduce our initial co-IP experim ents, so have not c arried out additional experiments to lo calize any c omplexes containing MP1 and ER. However, our results continue to point to a functional relationship between these two proteins.

Task 3: Purify ER/MP1 complexes by sequential affinity purification and examine complex components by Western blotting.

Since we have been unable to reproduce our preliminary co-IP experim ents, we have concentrated our efforts on studying the role of MP1 in breast cance r cells, as described in Task 1 above.

Task 4: Identify novel components of ER/MP1 complexes by mass spectrometry.

The experiments described in this task are dependent on purifying ER/MP1 complexes. As described under Task 2 and 3, we were unable to accomplish this.

Key Research Accomplishments:

- Demonstrated that MP1 expression is not required for survival of non-tumorigenic mammary epithelial cell lines.
- Demonstrated that knock-down of MP1 leads to decreased AKT phosphorylation. Initiated experiments to identify additional signaling molecules whose expression or activity is dependent on MP1 expression in ER-positive breast cancer cells.
- Demonstrated that overexpression of MP1 increases cell migration and invasion of ER-positive MCF-7 cells.
- Demonstrated that knock-down of MP1 does not decrease migration of ER-negative MDA-MB-231 cells.

Reportable Outcomes:

1) Poster presentation at Great Lakes Nuclear Receptor Meeting.

Conclusion: Our results have revealed a novel role for MP1 in the survival of ER-positive breast cancer cell lines. Blocking MP1 expression using siRNA leads to apoptotic cell death in ER-positive MCF-7, LCC9 and T47D cells, but not in ER-negative MDA-MB-231, SKBr3 and BT-549 breast cancer cells, or in non-tumorigenic 184B5 cells. The apoptosis observed after MP1 knockdown in MCF-7 cells is correlated with decreases in both AKT phosphorylation and ER expression, and we hypothesize that a loss of pro-survival signaling from one or both of these molecules may contribute to cell death. We are in the process of identifying additional molecules/pathways that may play a role in the pro-survival functions of MP1. The current targeted therapies for ER-positive breast tumors (antiestrogens and aromatase inhibitors) induce a cell cycle arrest, but do not rapidly kill tumor cells. The fact that tumor cells can survive for long periods of time in the presence of aromatase inhibitors and antiestrogens may provide the opportunity for resistance to develop. Novel therapeutics with the ability to kill ER-positive tumor cells would offer several potential advantages, including decreased treatment time and decreased development of resistance. At the moment, there is not a well-established approach to targeting scaffold proteins such as MP1. However, such approaches may be identified in the future. In addition, if we are successful in identifying the pathways involved the pro-survival function of MP1, they may provide more traditional therapeutic targets.

In addition to its role in cell survival, our results suggest that high levels of MP1 expression increase migration and invasion in ER-positive breast cancer cells. Since both of these are associated with more aggressive tumors, high levels of MP1 expression in ER-positive tumors could be an indicator of a poorer prognosis.

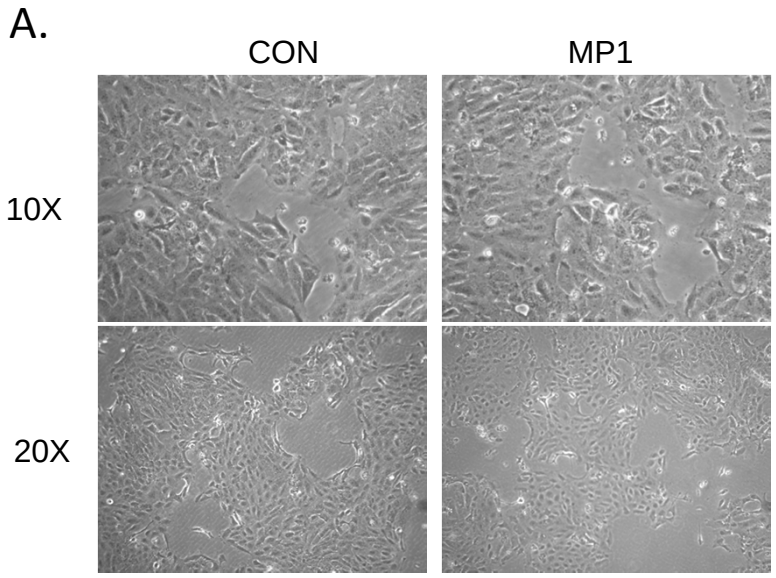
References:

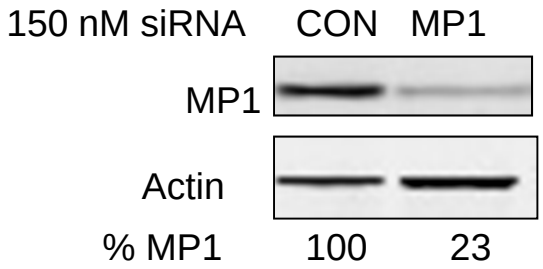
1. Schaeffer, H.J., et al., *MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade.* Science, 1998. 281(5383): p. 1668-71.
2. Pullikuth, A., et al., *The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals.* Mol Cell Biol, 2005. 25(12): p. 5119-33.
3. Park, E.R., et al., *Differential requirement for MEK Partner 1 in DU145 prostate cancer cell migration.* Cell Commun Signal, 2009. 7: p. 26.

Appendix/Supporting Data:

Figures 1-5

Abstract from Great Lakes Nuclear Receptor Meeting





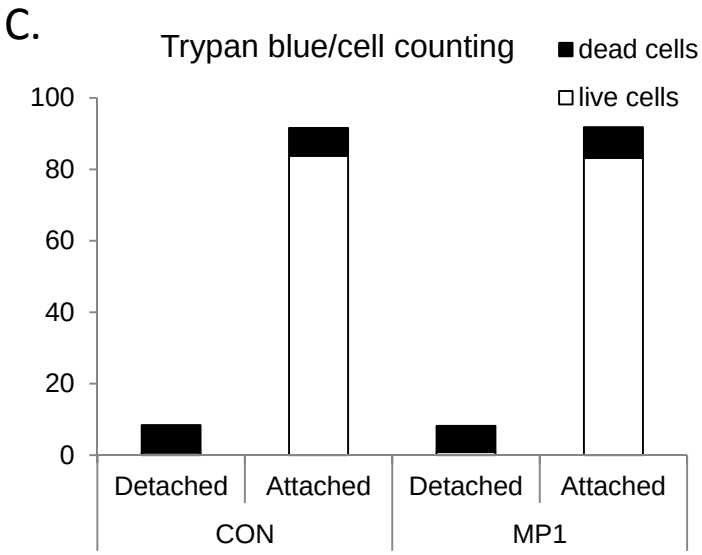


Figure 1: Effect of MP1 knockdown on non-tumorigenic mammary epithelial cells. Non-tumorigenic 184B5 cells were transfected with control or MP1 siRNA and analyzed at 8 h after transfection. A) Photographs of cells. B) Western blots of cell extracts showing MP1 knockdown. C) Both attached and detached cells were harvested, stained with trypan blue, and counted.

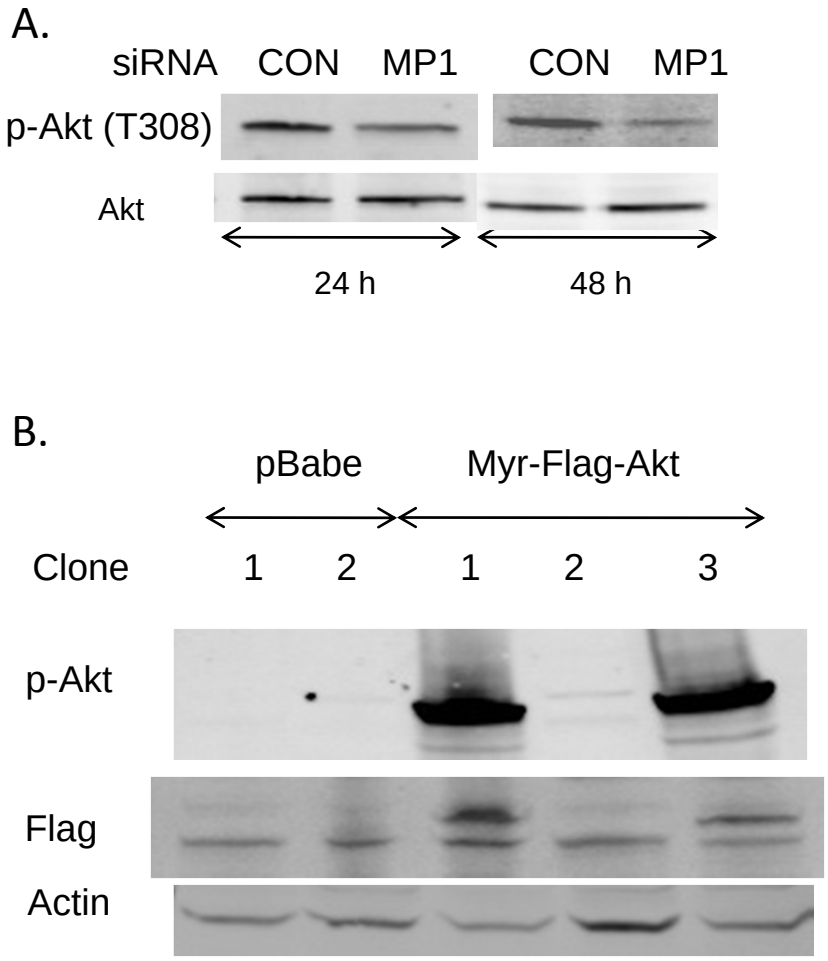
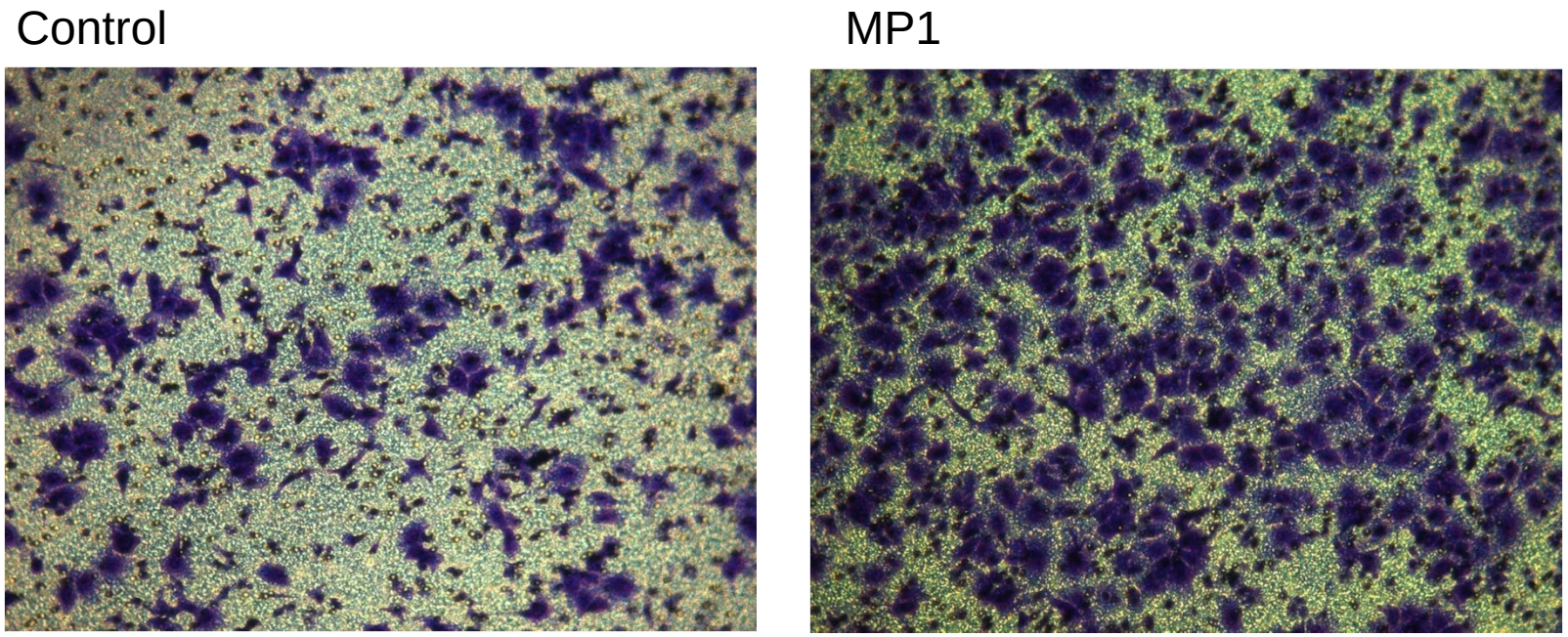


Figure 2. Effect of MP1 knockdown on AKT activity in MCF-7 cells. A) MCF-7 cells were transfected with control or MP1 siRNA. Cell extracts were prepared at 24 and 48 h, and analyzed for the levels of total and phosphorylated AKT by western blotting. B. MCF-7 cells were transfected with pBabe vector or a pBabe construct encoding a Flag-tagged constitutively actived (myristylated) form of AKT1 (Myr-Flag-AKT). Stable transfectants were selected, and analyzed for phospho-AKT, Flag-AKT, and actin by western blotting. Myr-Flag-AKT clones 1 and 3 are expressing the transfected gene.



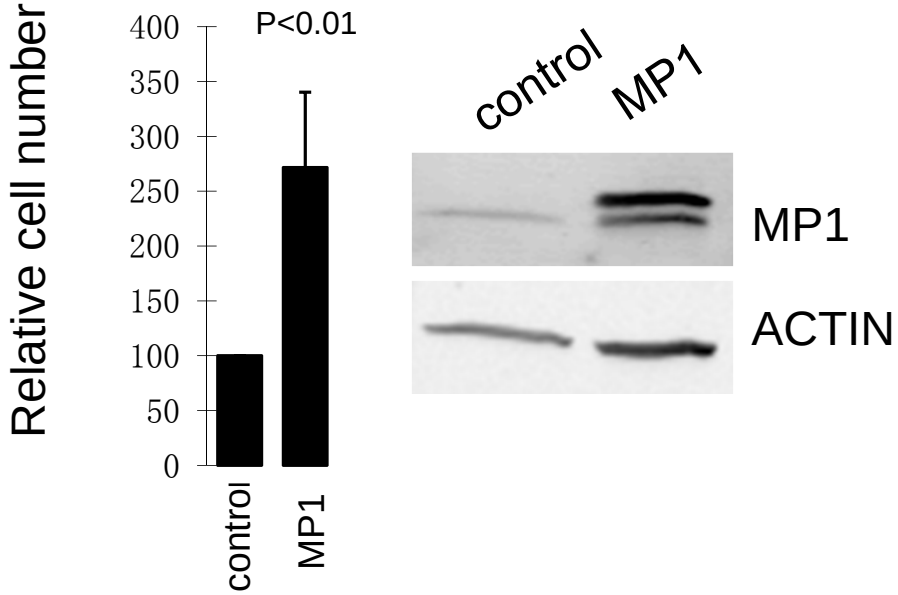
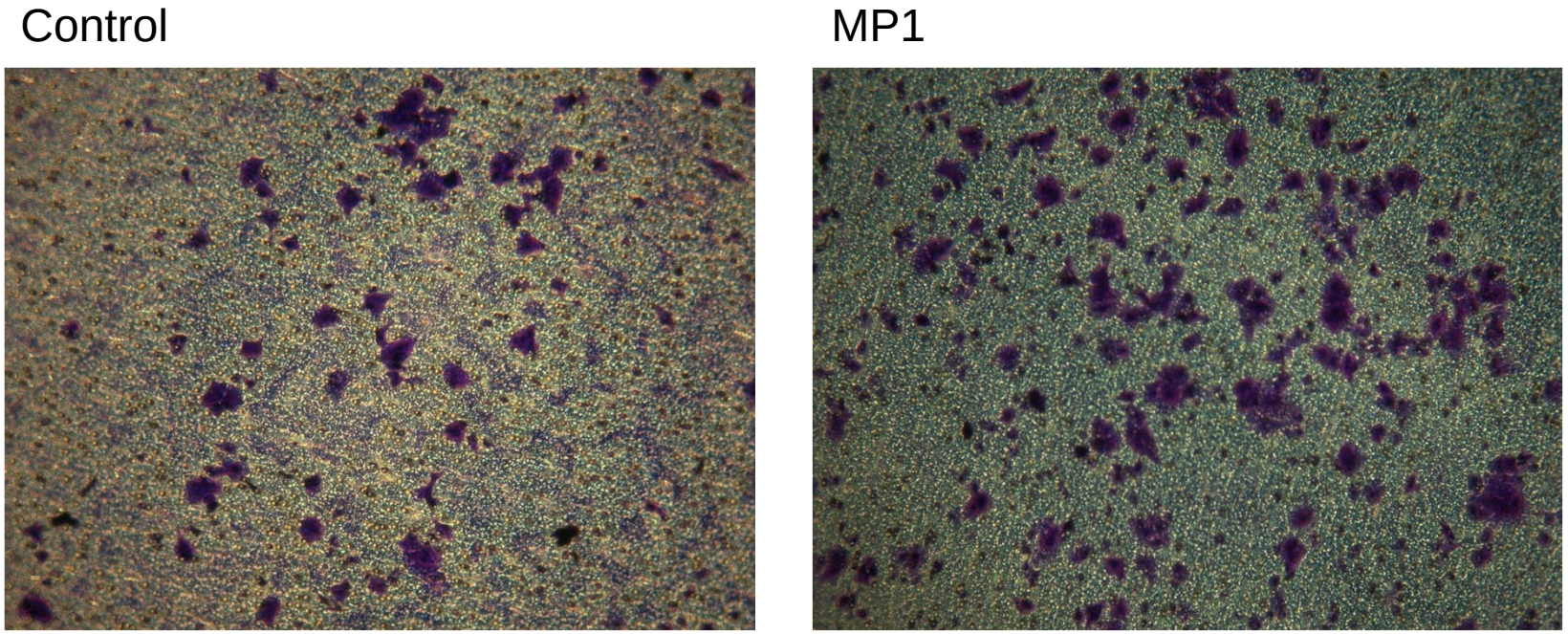


Figure 3. Effects of MP1 overexpression on cell migration. MCF-7 cells were transfected with pCMV-Flag-MP1 or pCMV vector, then serum starved overnight. For cell migration assays 1X10^{5}cells in serum free medium were added to the upper well of the chamber, and medium containing 10% serum was added to the lower chamber. All media contained mitomycin C to inhibit proliferation. Cells on the bottom of the filter were fixed and stained after 24 h, then counted. Top panel: photographs of stained cells. Bottom panel: Quantitation of migrated cells, normalized to control (100%), N=3. Western blot confirming MP1 overexpression.



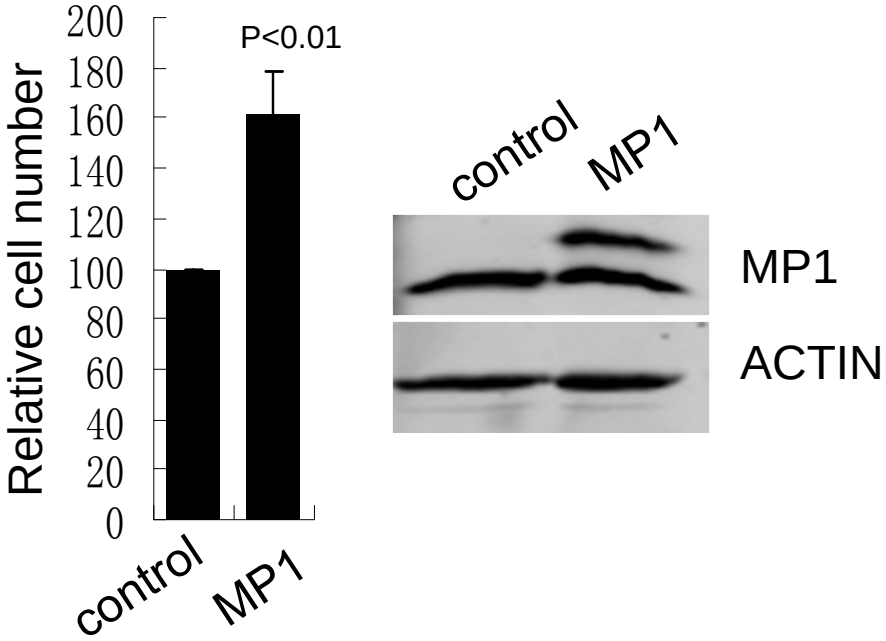


Figure 4: Effects of MP1 overexpression on cell invasion. Experiment was performed as described in Figure 3, except that the upper well was coated with matrigel.

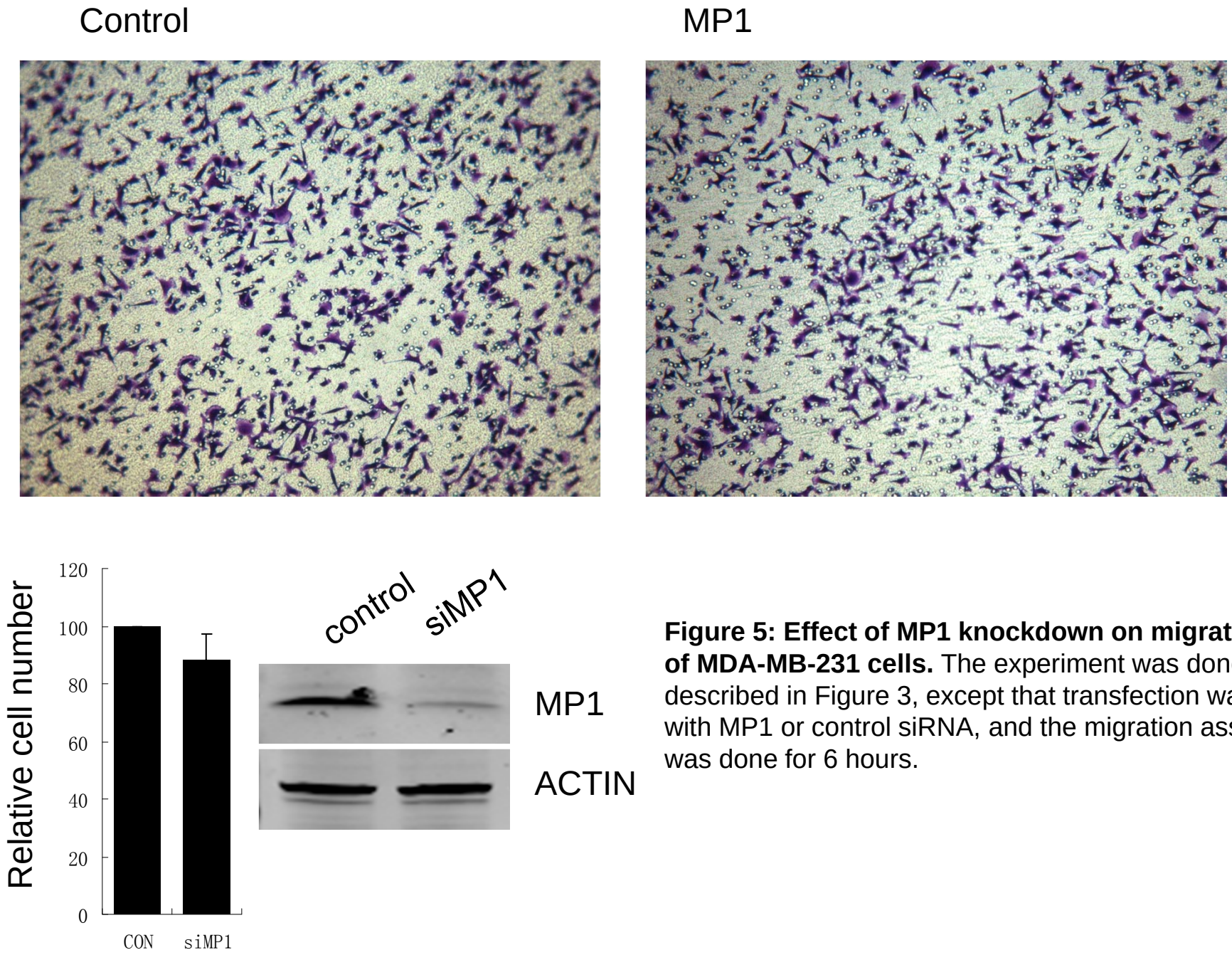


Figure 5: Effect of MP1 knockdown on migration of MDA-MB-231 cells. The experiment was done as described in Figure 3, except that transfection was with MP1 or control siRNA, and the migration assay was done for 6 hours.

INHIBITION OF MP1 EXPRESSION INDUCES APOPTOSIS OF ER-POSITIVE BREAST CANCER CELLS

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Estrogen receptor (ER) plays a prominent role in breast cancer biology. Approximately 70% of breast tumors express ER, and the majority of these require estrogen for proliferation and/or survival. Many functions of the ER rely on cross-talk with cellular signaling molecules, including MAPK, PAK1, and AKT1. These intracellular kinases have all been reported to phosphorylate ER, and these phosphorylation events may alter its activity and/or ligand dependence. In addition, ER may affect the activity of these intracellular kinases via protein-protein interactions with upstream signaling molecules. Understanding the molecular bases and functional consequences of cross talk between ER and other cellular signaling pathways is therefore important for a complete understanding of its role in breast cancer. MP1 (MEK Partner 1, also known as MAPK scaffold protein 1 or MAPKSP1) is a widely expressed scaffold protein that functions in several signaling pathways (MAPK and PAK1) that are known to impact ER function and breast cancer biology. We therefore hypothesized that MP1 might play an important role in ER positive breast cancer cells. To test this hypothesis, we have investigated the function of MP1 in a representative panel of human mammary epithelial cell lines: 184B5 (non-tumorigenic, ER-negative), MCF-7 (ER-positive, estrogen dependent and antiestrogen sensitive) and MDA-MB-231 (ER- negative). The effects of inhibiting MP1 expression by transient transfection with siRNA duplexes were examined. After 48 hours, MP1siRNA-treated MCF-7 cells displayed evidence of cell death, but this effect was absent in control siRNA-treated MCF-7 cells and in both control and MP1siRNA-treated 184B5 and MDA-MB-231 cells. Cell counting and trypan blue exclusion indicated that approximately 80% of MCF-7 cells treated with MP1 siRNA rounded up and detached from the plate, with a majority of detached cells being trypan blue-positive. In the ER-negative cell lines, no significant increase in cell death was observed in cells treated with MP1 siRNA relative to control siRNA. Protein immunoblotting confirmed that MP1 protein levels were successfully reduced in all cell lines. Consistent with the cell death phenotype, cleavage of PARP-1 protein was detected only in MP1 siRNA treated MCF-7 cells, suggesting that knockdown of MP1 causes apoptosis in this cell line. This was confirmed using the pan-caspase inhibitor z-VAD-FMK, which rescued the cell death phenotype. Furthermore, the apoptosis of MCF-7 cells was correlated with decreased levels of ER protein and mRNA, as well as decreased p-Akt protein levels. These results suggest that the inhibition of MP1 expression resulted in a loss of pro-survival signaling from the ER and/or PI3K-AKT pathway. Finally, several additional ER-positive and ER-negative breast cancer cell lines have been examined, and the results are consistent with a model in which MP1 expression is specifically required for survival of ER-positive cells.

Preferred Session: Development and Disease